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Comparative analysis of metagenomics between high- and medium-temperature daqu, and microbial succession in Jiang-Nong Jianxiang Baijiu fermentation

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Abstract

Background The mixture of high-temperature Daqu and medium-temperature Daqu can be used to produce Chinese Jiang-Nong Jianxiang Baijiu. This study used metagenomic sequencing and physicochemical analysis to investigate the microbial community and functionality of high-temperature Daqu and medium-temperature Daqu. In addition, exploring the changes of microbial communities during the Jiang-Nong Jianxiang Baijiu fermentation process.

Results The results showed that *Lichtheimia ramose* and *Saccharopolyspora rectivirgula* were the significantly different species in high-temperature Daqu. However, *Paecilomyces variotii*, *Aspergillus chevalieri*, and *Rasamsonia emersonii* were the significantly different species in medium-temperature Daqu. The medium-temperature Daqu had higher saccharifying power (101.20 ± 1.85 U/g) than high-temperature Daqu (60.00 ± 0.58 U/g). And the protease activity of high-temperature Daqu (62.47 ± 5.84 U/mg) was significantly higher than medium-temperature Daqu (36.10 ± 1.13 U/mg). The community structure analysis results of the stack fermentation stage showed that the mixture of high-temperature Daqu and medium-temperature Daqu inherited the community advantages of both high-temperature Daqu and medium-temperature Daqu. With Jiang-Nong Jianxiang Baijiu fermentation, the significantly different species changed from *Pichia* sp., *Acetobacter* sp., and *Lactobacillus* sp. to *Pediococcus* sp., *Lactobacillus* sp., *Lentilactobacillus* sp., *Saccharomyces* sp., *Thermoactinomyces* sp., and *Saccharopolyspora* sp., implying the importance of acid-resistant and ethanol-resistant microorganisms for the production of flavor substances in the late Baijiu fermentation.

Conclusions Our research revealed the difference in microbial communities between high-temperature Daqu and medium-temperature Daqu, and demonstrated the shifts and functionality of microbiota during Jiang-Nong Jianxiang Baijiu fermentation. This study provides a theoretical reference for utilizing core synergistic microbiota and their functional traits in Baijiu fermentation starters to improve Baijiu quality.

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Keywords Daqu, Jiang-Nong jianxiang baijiu, Compound-Flavor baijiu, Metagenomic sequencing, Microbiota

Background

Chinese Baijiu is a renowned distilled spirit globally, distinguished by its long history and unique flavor profile [1]. Serving as the saccharifying agent, fermentation starter, and raw material for Baijiu production, Daqu (a traditional Chinese fermentation starter) is categorized by fermentation temperature into high-temperature, medium-temperature, and low-temperature types. High-temperature Daqu production involves a unique process where temperatures progressively rise above 60 °C [2]. In contrast, medium-temperature and low-temperature Daqu are incubated within ranges of 50 °C to 60 °C and 45 °C to 50 °C, respectively [3]. Beyond supplying diverse microorganisms and enzymes essential for fermentation, Daqu significantly influences the quality of the final Baijiu product [4]. However, Daqu is susceptible to variations in the open production environment, specific techniques, and raw materials [5]. Therefore, understanding the differences in microbial community structure and flavor characteristics among different Daqu types, including mixed Daqu, is crucial for Baijiu fermentation research.

To meet growing consumer demand for new tastes and health-conscious consumption, researchers are actively exploring ways to optimize Baijiu quality. Therefore, using mixed Daqu starters to yield varied flavor profiles has garnered research interest. The mixture of high-temperature Daqu and medium-temperature Daqu can be used to produce Jiang-Nong Jianxiang Baijiu, which is one type of category in compound-flavor Baijiu and combines the characteristics of both Jiang (sauce)-flavored and Nong (strong)-flavored types of Chinese Baijiu. However, the produce process of Jiang-Nong Jianxiang Baijiu primarily relies on empirical knowledge. It remains unclear how to specifically optimize the microbial community structure during mixed fermentation to enhance the brewing quality and taste of Jiang-Nong Jianxiang Baijiu. To address this challenge, this study aims to analyze the microbial community composition of the starter at each fermentation stage. This analysis will provide a foundation for subsequent Baijiu quality optimization, facilitating the selection of dominant strains or enabling targeted regulation of key genes.

Traditional microbial culture techniques provide insights into the relationship between cultivable microorganisms and flavor compounds in Baijiu production [6]. For instance, *Clavispora lusitaniae*, isolated from Daqu in the laboratory, could synthesize ethyl caproate, ethyl acetate, ethyl lactate, ethyl butyrate, and ethyl octanoate to enhance the quality of Baijiu [7]. Similarly, *Lactobacillus plantarum*, *Weissella paramesenteroides*, and *Pediococcus pentosaceus*, selected and inoculated from low-temperature Daqu in the laboratory, could produce ethyl lactate, ethyl acetate, tetramethylpyrazine, and 4-ethyl-2-methoxyphenol to significantly improve Baijiu quality [8]. However, laboratory cultivation conditions disrupt natural microbial interactions, reducing cultivability [9]. This limitation hinders comprehensive understanding of microbial diversity, composition, and functional dynamics within Baijiu fermentation starters. In recent years, using metagenomics to decode microbial composition, interactions, metabolic pathways, and gene functions at molecular resolution during food fermentation processes has become a trend [10, 11]. Huang et al. (2025) elucidated the impact of viruses on shaping microbial composition and influencing metabolic functions in Daqu by metatranscriptomic analysis [12]. Ren et al. (2025) revealed microbial community and functional differences between three different grades of Hongxin low-temperature Daqu by metagenomics to verify the different fermentation effects of different grades of Hongxin low-temperature Daqu in light-flavor Baijiu [13]. Yang et al. (2024) employed metagenomics to analyze the microecological succession of high-temperature Daqu during storage, elucidate the adaptation mechanism of the microbial community of Daqu to storage environments, and clarify the microecological characteristics of Daqu during different seasons [14]. Therefore, Utilizing metagenomics to elucidate the microbial composition of high-temperature Daqu and medium-temperature Daqu is important for optimizing the quality of Jiang-Nong Jianxiang Baijiu. Furthermore, applying metagenomics to reveal the changes of microbial and functional profiles in the fermentation processes of Jiang-Nong Jianxiang Baijiu has been reported in few studies.

This study utilized metagenomic sequencing to investigate differences in microbial composition and functional profiles between high-temperature Daqu and medium-temperature Daqu. Furthermore, it employed the same metagenomic approach to track temporal changes in microbial communities and functional gene expression throughout the fermentation stages of Jiang-Nong Jianxiang Baijiu. In order to clarify the microecological characteristics of fermentation material during different Jiang-Nong Jianxiang Baijiu fermentation stages. This analysis will provide theoretical reference for optimizing Baijiu quality and facilitating the selection of dominant strains.

Methods

Sample collection

Medium-temperature Daqu samples (Za, Zb, Zc), high-temperature Daqu samples (Ga, Gb, Gc), stack

fermentation material samples (gwa, gwb, gwc), and fermented grain samples (zga, zgb, zgc) were procured from Yupo Baijiu Co., located in Zhumadian, Henan, China (31°23'–36°22'N, 110°21'–116°39'E) (Fig. 1). The stack fermentation materials were produced by blending high-temperature Daqu and medium-temperature Daqu at a ratio of 1.8:1 with sorghum and other raw materials, followed by a two-day stacking period. The fermented grain samples were obtained after these stack fermentation materials underwent a 60-day fermentation process in standard fermentation chambers. The medium-temperature Daqu and high-temperature Daqu samples were collected in October 2023. When stack fermentation material samples were collected, the temperature and the humidity of the environmental were 32–36 ℃ and 58–62%, respectively. When fermented grain samples were collected, the temperature and the humidity of the environmental were 25–26 ℃ and 64–68%, respectively. Three high-temperature Daqu samples were collected from the upper, middle, and lower layers of a high-temperature Daqu stack. Three medium-temperature Daqu samples were similarly collected from the upper, middle, and lower layers of a medium-temperature Daqu stack. Three random points were selected in the stack fermentation materials, and stratified samples (upper, middle, and lower layers) were retrieved from each point and pooled to form one composite sample per point. Three random pits were selected, and stratified fermented grain samples (upper, middle, and lower layers) were retrieved from each pit and pooled to form one composite sample per pit. All samples were cryopreserved at −80 °C for metagenomic sequencing or −20 °C for physicochemical analyses.

Fig. 1 Sample categories and processing stages: (**G**) high-temperature Daqu, (**Z**) medium-temperature Daqu, (**gw**) blended high-temperature Daqu and medium-temperature Daqu at a 1.8:1 ratio with sorghum and other raw materials after two-day stack fermentation, (**zg**) pit-fermented mixture following 60-day fermentation

Physicochemical properties

Water content was determined gravimetrically by drying samples at 80 °C for 4 h until constant weight. Dry matter content was derived directly from water content measurements. Esterification, saccharification, and liquefaction powers were assayed following Method [15]. Esterification power was quantified as ethyl caproate production (mg) from 1 g sample incubated at 30–32 °C for 100 h. Saccharification power (U) was expressed as glucose yield (mg) from soluble starch hydrolysis by 1 g sample at 35 °C (pH 4.6) per hour. Liquefaction power (U) represented soluble sugar production (mg) from soluble starch hydrolysis by 1 g sample at 60 °C (pH 4.6) per hour. Protease activity was determined according to [16], with one unit (U) defined as tyrosine release (1 g) from casein hydrolysis by 1 g sample per minute at 40 °C. All analyses were performed in triplicate.

DNA extraction and metagenomic sequencing

Genomic DNA was extracted from all samples using the Guan et al. method [17]. DNA purity and integrity were verified by 1% agarose gel electrophoresis (AGE), with concentrations quantified via Qubit 2.0 Fluorometer (Qubit 2.0, Life Technologies). Subsequently, 1 μg of DNA per sample was fragmented to ~350 bp using a Covaris S220 ultrasonicator. Library preparation involved end repair, A-tailing, adapter ligation, purification, and PCR amplification. Prepared libraries underwent Qubit quantification (Qubit 2.0, Life Technologies) and dilution to 2 ng·μL^{-1}, followed by insert size verification on an Agilent 2100 Bioanalyzer. Quantitative PCR determined effective library concentrations before pooling based on desired sequencing depth. Libraries were sequenced on Illumina platforms (PE150). Raw reads were processed with Readfq (GitHub: cjfields/readfq) to generate clean data, followed by metagenomic assembly using MEGAHIT v1.2.9 [18].

Gene prediction and abundance analysis

Open reading frames (ORFs) were predicted from scaftigs (≥500 bp) using MetaGeneMark (http://topaz.gatech.edu/GeneMark/) [19]. Predicted ORFs < 100 nucleotides in length were excluded. CD-HIT (http://www.bioinformatics.org/cd-hit/) [20] dereplicated these ORFs to generate a non-redundant gene catalog [21]. Clean reads were aligned to this catalog via Bowtie2 (v2.1.0), with gene-specific read counts quantified [19]. Genes with ≤2 aligned reads per sample were filtered to yield the final Unigenes catalog [21]. Gene abundance was calculated based on aligned read counts and gene length [22]. This

catalog enabled: (a) basic statistical analyses, (b) core/pan-genome assessment, (c) inter-sample correlations, and (d) gene distribution profiling via Venn diagrams.

Species annotation

Unigenes were taxonomically annotated by aligning against bacterial, fungal, archaeal, and viral sequences from NCBI's NR database using DIAMOND (v4.4.1) [23]. Species assignments were subsequently determined via the lowest common ancestor (LCA) algorithm e [24].

Common functions database annotation

Functional annotation of Unigenes was performed against KEGG, eggNOG, and CAZy databases using DIAMOND (v4.4.1) [19], retaining the highest-scoring alignments for downstream analysis. Relative abundance profiles were computed across functional hierarchies. Taxonomic abundance matrices were generated by integrating annotation results with gene abundance data. Subsequent analyses comprised: relative abundance profiling with clustered heatmaps; dimensionality reduction via PCA and NMDS; inter/intra-group differentiation assessment using Anosim; and Metastat/LEfSe-based identification of differential functions across taxonomic levels.

Resistance gene annotation

Unigenes underwent functional annotation against the Comprehensive Antibiotic Resistance Database (CARD) via Resistance Gene Identifier (RGI) software. Antibiotic Resistance Ontology (ARO) abundances were quantified by integrating RGI outputs with unigene abundance profiles. Subsequent analytical phases encompassed: visualization of ARO distributions through abundance bar charts, clustered heatmaps, and circos plots; differential abundance assessment of resistance genes across experimental groups; and taxonomic attribution analysis of resistance mechanisms.

Results and discussion

Microbial composition based on metagenomics

At the kingdom level, high-temperature Daqu, medium-temperature Daqu, and stack fermentation material, were mainly composed of bacteria and eukaryotes, and their total relative abundance were over 70% (Fig. S1). This is due to the fact that bacteria and eukaryotes are important microorganisms producing various enzymes, ethyl alcohol, and aroma substances in Daqu [25]. However, the bacterial richness of high-temperature Daqu was higher than that of medium-temperature Daqu (Fig. S1). It is easier for bacteria to grow and reproduce at high-temperatures, while it is relatively difficult for eukaryotes to survive in such an environment [5]. Therefore, high temperature culture increased the abundance proportion of bacteria. At the phylum level, the microbiota structure of stack fermentation material showed high similarity with high-temperature Daqu and medium-temperature Daqu. In medium-temperature Daqu, 3 phyla showed average relative abundances above 0.9% (Fig. 2A), namely Ascomycota (52.81%), Bacillota (12.83%), and Mucoromycota (2.30%), respectively. In high-temperature Daqu, 5 phyla showed average relative abundances above 0.9% (Fig. 2A), namely Ascomycota (29.10%), Actinomycetota (17.49%), Bacillota (12.23%), Mucoromycota (10.59%), and Pseudomonadota (2.57%), respectively. These results illustrated that high-temperature Daqu has more abundant high-abundance strains than medium-temperature Daqu, which may produce more flavor substances. In stack fermentation material, 4 phyla showed average relative abundances above 0.9% (Fig. 2A), namely Ascomycota (57.27%), Pseudomonadota (23.02%), Bacillota (2.81%), and Actinomycetota (1.02%), respectively. These results indicated that the mixture of high-temperature Daqu and medium-temperature Daqu inherited the high Actinomycetota proportion of medium-temperature Daqu and the species richness of high-temperature Daqu.

Medium-temperature Daqu featured two predominant genera: *Aspergillus* (15.33%) and *Pediococcus* (1.65%), both exceeding 0.9% average relative abundance (Fig. 2B). High-temperature Daqu contained three major genera: *Saccharopolyspora* (15.71%), *Aspergillus* (6.40%), and *Pediococcus* (1.70%) at similar abundance thresholds. At the species level, medium-temperature Daqu was dominated by *Paecilomyces variotii* (10.00%) and *A. chevalieri* (4.97%), while high-temperature Daqu showed prominence of *S. rectivirgula* (13.94%), *P. variotii* (4.80%), and *A. chevalieri* (0.91%) (Fig. 2C). *Aspergillus* and *Pediococcus* serve as crucial saccharification catalysts in starch-based Baijiu production and contribute significantly to flavor development, particularly of pyrazines, esters, and aromatic compounds [26, 27]. *P. variotii* and *A. chevalieri* were abundant in both Daqu types, whereas *Saccharopolyspora* demonstrated markedly higher abundance in high-temperature Daqu. This genus generates bioactive peptides, oligosaccharides, and diverse enzymes that enhance fermentation [28]. Notably, *S. rectivirgula* emerged as the predominant *Saccharopolyspora* species in high-temperature Daqu, suggesting its potential role as a primary flavor precursor in Nongxiang-style Baijiu [29].

After two days of stack fermentation, three dominant genera exceeding 0.9% average relative abundance were observed: *Pichia* (54.07%), *Acetobacter* (1.80%), and *Lactobacillus* (0.96%) in the stack fermentation material, significantly higher than in medium- and high-temperature Daqu (Fig. 2B). Conversely, *Aspergillus* (0.13%), *Pediococcus* (0.21%), and *Saccharopolyspora* (0.72%) showed substantially reduced abundances compared to Daqu samples. This differential abundance profile indicates

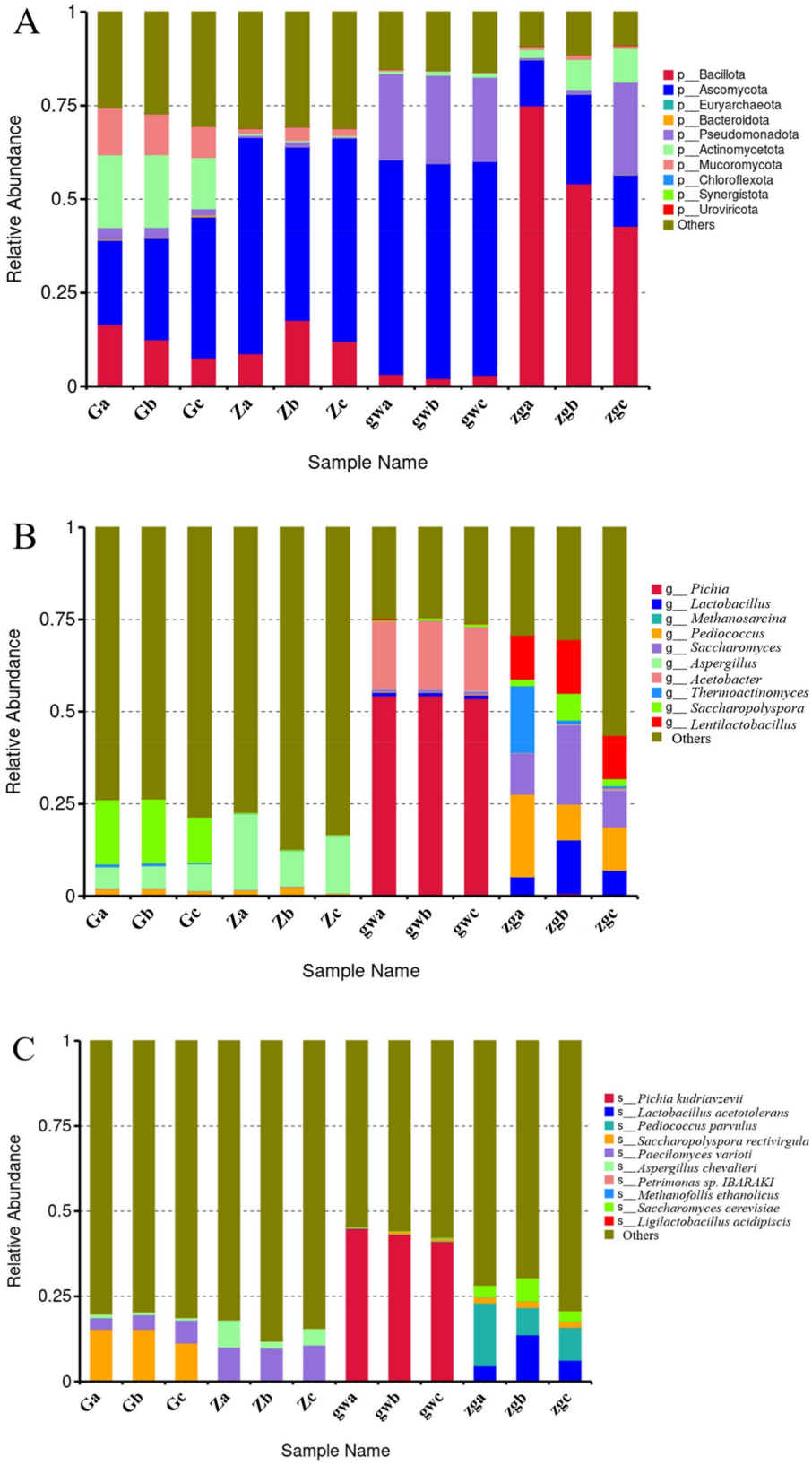

Fig. 2 Microbial composition at the phylum (**A**), genus (**B**), and species (**C**) levels across sample types: high-temperature Daqu (Ga, Gb, Gc), medium-temperature Daqu (Za, Zb, Zc), stack fermentation material samples (gwa, gwb, gwc), and fermented grain samples (zga, zgb, zgc)

that the mixture of high-temperature Daqu and medium-temperature Daqu retains microbial characteristics from both parent Daqu types. *Lactobacillus* and *Acetobacter* generate lactic and acetic acids-key non-volatile organic acids shaping Baijiu's flavor profile. Their dominance elevates acidity during fermentation, selectively inhibiting non-acid-tolerant microorganisms. At the species level, *Pichia kudriavzevii* (43.07%) emerged as the sole taxon exceeding 0.9% abundance in the stack fermentation material (Fig. 2C). As the primary ethanol and ethyl acetate producer in Baijiu [30], its predominance reflects the unique stack fermentation material environment's enhanced suitability for this species.

In order to identify the major contributing strains of flavor substances and community structure changes during Jiang-Nong Jianxiang Baijiu fermentation process, the community structure in fermented grain (the stack fermentation materials fermented in the standard fermentation chambers for 60 days) was examined. At the genus level, there were 6 genera with average relative abundances above 0.9% in fermented grain, namely *Pediococcus* (14.60%), *Saccharomyces* (14.24%), *Lentilactobacillus* (12.69%), *Lactobacillus* (8.76%), *Thermoactinomyces* (6.58%), and *Saccharopolyspora* (3.67%), which differed from the stack fermentation stage (Fig. 2B). Similar results were detected at the species level, with 4 species showing average relative abundances above 0.9% in fermented grain (Fig. 2C), namely *Pediococcus parvulus* (12.05%), *Lactobacillus acetotolerans* (8.11%), *Saccharomyces cerevisiae* (4.55%), and *S. rectivirgula* (1.77%). This may be due to the fact that the stack fermentation stage is dominated by starch saccharification, alcohol production, and acid production, while the final fermentation stage (fermented grain) is dominated by ethanol-tolerant bacteria, acid-resistant, and flavor-producing bacteria. Furthermore, these results also illustrated that *Pediococcus* (*Pediococcus parvulus*) [8], *Saccharomyces* (*Saccharomyces cerevisiae*) [31], *Lentilactobacillus* [32], *Lactobacillus* (*Lactobacillus acetotolerans*) [8], *Thermoactinomyces* [25], and *Saccharopolyspora* (*Saccharopolyspora rectivirgula*) [28] contribute significantly to flavor substances production such as ethanol, acetic acid, lactic acid,1,2-propanediol, ethyl acetate, ethyl lactate, ethyl propionate, ethyl nonanoate, ethyl palmitate, ethyl dodecanoate, ethyl benzoate, tetramethyl-pyrazine, 4-ethyl-2-methoxy-phenol, peptides, and oligosaccharides in Baijiu.

Differences in the microbial community structure

To visualize the differences in bacterial community structure among four sample groups, various statistical techniques were employed: Principal Component Analysis (PCA), Non-Metric Multi-Dimensional Scaling (NMDS), and Principal Co-ordinates Analysis (PCoA) based on the Bray-Curtis metric, as well as Cluster analysis using the Bray-Curtis distance. The proximity of samples in these visualizations indicates the similarity of their species composition; samples with highly similar communities are closer together, while those with vastly different communities are farther apart. The results from PCoA based on Bray-Curtis distance, PCA, and NMDS collectively demonstrated that the species composition structure of the four sample groups was distinctly separable (Fig. S2).

To identify significant differences in the microbial communities of high-temperature Daqu and medium-temperature Daqu, and significant variations in the microbial communities of Baijiu fermentation process, the LEfSe method was conducted (Fig. 3). The microbiota cladogram results showed that there were significant differences in the composition of microbial communities between medium-temperature Daqu and high-temperature Daqu (Fig. 3A), and significant differences between stack fermentation material and fermented grain (Fig. 3C). Therefore, we analyzed the significantly different species between medium-temperature Daqu and high-temperature Daqu (Fig. 3B), as well as between stack fermentation material and fermented grain (Fig. 3D).

Comparing the medium-temperature Daqu with the high-temperature Daqu, the significantly different species included *Lichtheimia ramose* and *S. rectivirgula* in high-temperature Daqu (Fig. 3B), while *Paecilomyces variotii*, *A. chevalieri*, and *Rasamsonia emersonii* were the significantly different species in medium-temperature Daqu (Fig. 3B). In high-temperature Daqu, *L. ramosa* has the potential to produce thermostable α-amylase and mannase, which are helpful for improving the efficiency of maltose and mannose production, respectively [33]. Maltose and mannose can promote the growth of Pichia and improve the taste of Baijiu. In addition to producing thermostable a-amylase, *S. rectivirgula* is also an important producer of flavor substances in Baijiu [34]. In medium-temperature Daqu, *Paecilomyces variotii* is involved in starch saccharification and the production of flavor substances [35]. *A. chevalieri* has the ability to secrete a range of enzymes, including amylase, protease, peptidase, and esterase [36]. These enzymes facilitate the breakdown and utilization of substrates found in raw materials, such as starch, proteins, and esters, thereby supplying the necessary precursors for the synthesis of flavor compounds. In addition to producing a-amylase, *Rasamsonia emersonii* produces aromatic amino acids [37] and fatty acids [38]. These results indicated that these significantly different species may be one of the reasons for the difference in taste between high-temperature and medium-temperature Daqu fermented Baijiu. Comparing the stack fermentation material with fermented grain, the significantly different species included *Pichia Candida californica*, *Acetobacter malorum*, *Acetobacter*

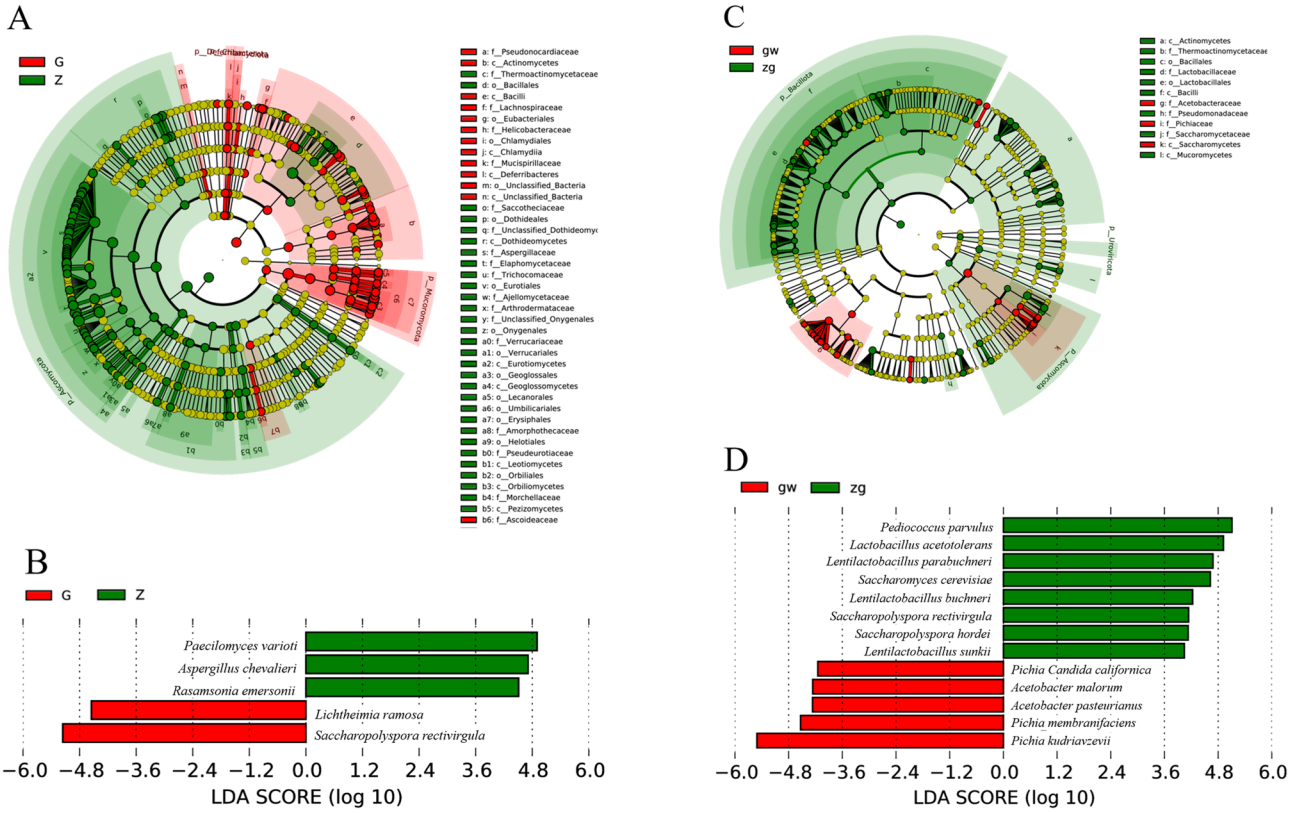

Fig. 3 LEfSe analysis of microbial community divergence: (**A**) Cladogram and (**B**) LDA score distribution for high-temperature (G) vs. medium-temperature Daqu (Z); (**C**) Cladogram and (**D**) LDA score distribution for stack fermentation material (gw) vs. fermented grain (zg). Cladograms (**A**, **C**) represent taxonomic hierarchies (phylum→species) where circle diameters scale with relative abundance. Yellow nodes indicate non-significant taxa; group-specific colors denote differentially abundant lineages

pasteurianus, *Pichia membranifaciens*, and *Pichia kudriavzevii* in stack fermentation material (Fig. 3D), while *Pediococcus parvulus*, *Lactobacillus acetotolerans*, *Lentilactobacillus parabuchnei*, *Saccharomyces cerevisiae*, *Lentilactobacillus buchneri*, *Saccharopolyspora rsctivirgula*, *Saccharopolyspora hordei*, and *Lentilactobacillus sunkii* were the significantly different species in fermented grain (Fig. 3D).

In stack fermentation material, except ethanol, *P. Candida californica*, *P. membranifaciens*, and *P. kudriavzevii* also can produce xylitol and phenylethyl alcohol, which can improve the Baijiu flavor [39]. *A. malorum* and *A. pasteurianus* can produce lactic acid, acetic acid, and D-gluconic acid [40, 41], which are the most important organic acid in Baijiu, can change the Baijiu body flavor. In fermented grain, *P. parvulus*, *L. acetotolerans*, *L. buchneri*, *L. sunkii*, and *L. parabuchnei* are capable of producing lactic acid and other small molecule compounds, which can endow the body unique flavor and taste, and increase the complexity of the Baijiu [42–44]. *S. cerevisiae* can produce ethyl acetate and isoamyl alcohol, which are important for baijiu flavor [45]. *S. rsctivirgula* and *S. hordei* can produce alcohols (phenylethanol, isoamylol and isobutanol), esters, amino acids (Pro, Arg, Glu and Ala) and organic acids (lactate, tartrate, acetate and citrate), which can endow the body unique flavor for baijiu [46]. These results showed that with Baijiu fermentation, the acid-resistant and ethanol-resistant microorganisms for the production of flavor substances in late Baijiu fermentation were important.

Functional gene category by blasting to cazy database

Principal Coordinates Analysis (PCoA) of CAZy levels 1 and 2 revealed distinct functional profiles across all four sample types (Fig. S3). Genes were classified into six CAZy categories: Glycoside Hydrolases (GH), Glycosyl Transferases (GT), Carbohydrate-Binding Modules (CBM), Auxiliary Activities (AA), Carbohydrate Esterases (CE), and Polysaccharide Lyases (PL) (Fig. 4A). Notably, GH enzymes dominated all samples, consistent with their critical roles in starch liquefaction, saccharification, and cellulose degradation [1]. GT and CBM families also demonstrated high relative abundance, aligning with their respective functions in oligosaccharide biosynthesis and substrate-enzyme binding enhancement for polysaccharide degradation [34]. However, compared to high-temperature Daqu and medium-temperature Daqu, GT had higher relative abundance in stack fermentation

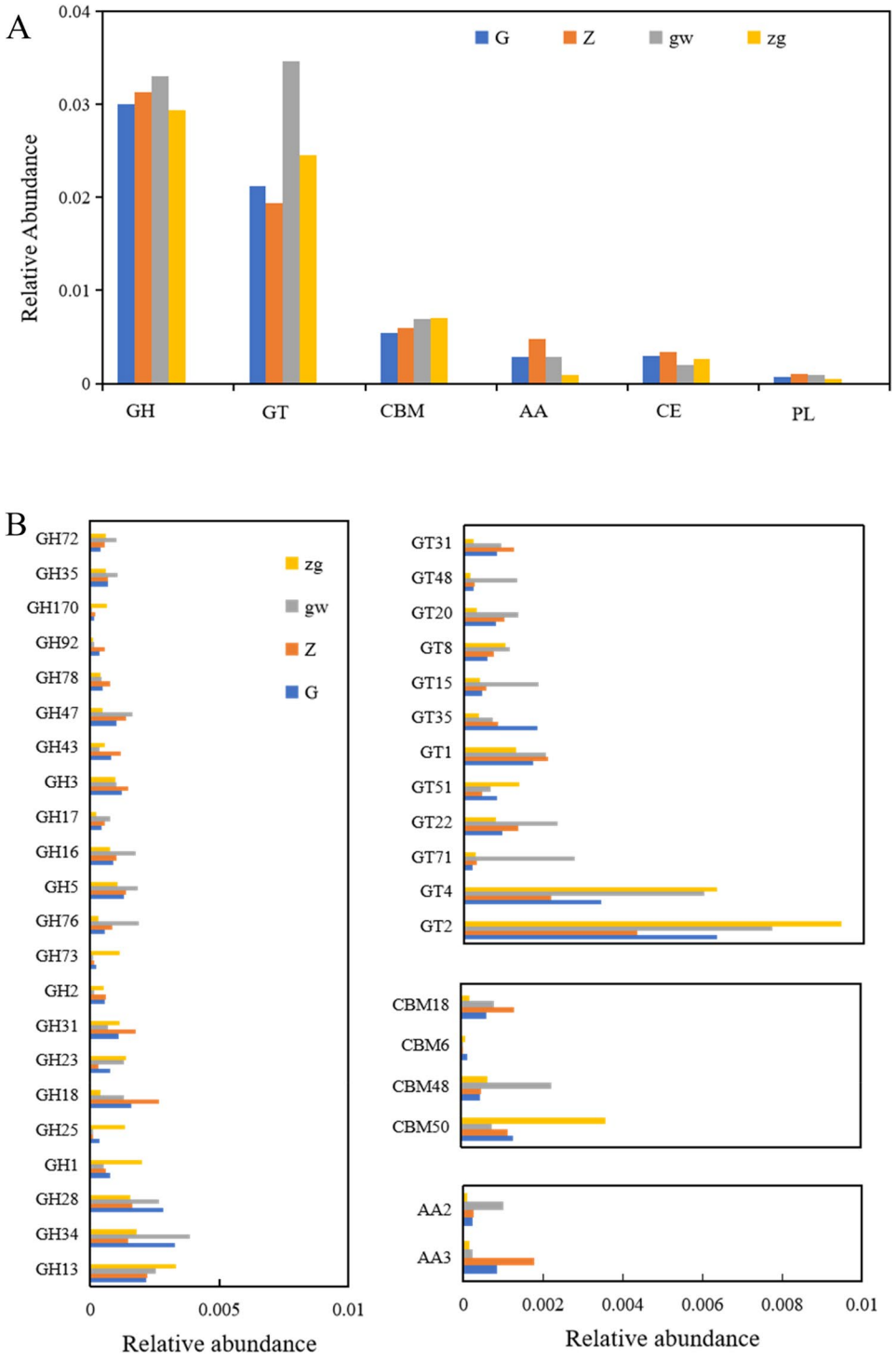

Fig. 4 CAZy functional annotation: **A** Enzyme class distribution (GH: Glycoside Hydrolases; GT: Glycosyl Transferases; CBM: Carbohydrate-Binding Modules; AA: Auxiliary Activities; CE: Carbohydrate Esterases; PL: Polysaccharide Lyases); **B** CAZy family profiles across sample types. Sample codes: G (high-temperature Daqu), Z (medium-temperature Daqu), gw (stack fermentation material), and zg (fermented grain)

material (Fig. 4A), which may be related to the fact that the microbial community is mainly involved in starch saccharification in the stack fermentation material.

Compared to the medium-temperature Daqu, the relative abundance of GH23, GH25, GH28, GH34, GT2, GT4, GT35, and GT51 were higher in high-temperature Daqu in CAZy families (Fig. 4B). CAZy-annotated GH23 and

GH25 lysozyme (EC 3.2.1.17) can directly degrade peptidoglycan, CAZy-annotated GH28 polygalacturonase (EC 3.2.1.15) can directly degrade pectate and other galacturonans, CAZy-annotated GH34 α-sialidase (EC 3.2.1.18) can directly degrade oligosaccharides, glycoproteins, glycolipids, colominic acid and synthetic substrates, CAZy-annotated GT2 cellulose synthase (EC 2.4.1.12) catalyzes the synthesis of cellulose, CAZy-annotated GT35 glycogen phosphorylase (EC 2.4.1.1) can directly degrade starch, CAZy-annotated GT4 α, α-trehalose synthase (EC 2.4.1.245) catalyzes the synthesis of trehalose, CAZy-annotated GT51 peptidoglycan glycosyltransferase (EC 2.4.99.28) catalyzes the synthesis of peptidoglycan (http://www.cazy.org/). Compared to the high-temperat ure Daqu, the relative abundance of GH18, GH31, GH3, GH43, GH47, GH78, GH92, GT31, GT1, and AA3 were higher in medium-temperature Daqu in CAZy families (Fig. 4B). CAZy-annotated GH18 chitinase (EC 3.2.1.14) can directly degrade chitin and chitodextrins, CAZy-annotated GH31 α-glucosidase (EC 3.2.1.20) can directly degrade starch, CAZy-annotated GH3 β-glucosidase (EC 3.2.1.21) can directly degrade cellulose, CAZy-annotated GH43 1,3-β-galactosidase (EC 3.2.1.145) can directly degrade galactan, CAZy-annotated GH47 and GH92 1,2-α-mannosidase (EC 3.2.1.113) can directly degrade mannosyl-oligosaccharide, CAZy-annotated GH78 α-L-rhamnosidase (EC 3.2.1.40) can directly degrade rhamnose, CAZy-annotated GT31 β–1,3-galactosyltransferase (EC 2.4.1.122) catalyzes the synthesis of oligosaccharides, CAZy-annotated GT1 β-glucosyltransferase (EC 2.4.1.35) catalyzes the synthesis of aryl β-D-glucoside, CAZy-annotated AA3 glucose 1-dehydrogenase (EC 1.1.5.9) catalyzes the formation of glucono-1,5-lactone (http://www.cazy.org/). The differences in the functions of these genes might explain why high-temperature Daqu and medium-temperature Daqu produce different flavors of Baijiu.

Compared to the fermented grain, the relative abundance of GH72, GH35, GH47, GH17, GH16, GH5, GH76, GH18, GH28, GH34, GT31, GT48, GT20, GT15, GT35, GT1, GT22, GT71, CBM18, CBM48, and AA2 were higher in stack fermentation material (Fig. 4B). CAZy-annotated GH35 1,3-β-galactosidase (EC 3.2.1.23) can directly degrade galactan, CAZy-annotated GH47 1,2-α-mannosidase (EC 3.2.1.113) can directly degrade mannosyl-oligosaccharide, CAZy-annotated GH16 and GH17 1,3-β-D-glucosidase (EC 3.2.1.39) can directly degrade glucan, CAZy-annotated GH5 cellulase (EC 3.2.1.4) can directly degrade cellulose, lichenin and cereal β-D-glucans, CAZy-annotated GH76 1,6-α-mannosidase (EC 3.2.1.101) can directly degrade mannan, CAZy-annotated GH18 chitinase (EC 3.2.1.14) can directly degrade chitin and chitodextrins, CAZy-annotated GH28 polygalacturonase (EC 3.2.1.15) can directly degrade pectate and other galacturonans, CAZy-annotated GH34 α-sialidase (EC 3.2.1.18) can directly degrade oligosaccharides, glycoproteins, glycolipids, colominic acid and synthetic substrates, CAZy-annotated GT1 β-glucosyltransferase (EC 2.4.1.35) atalyzes the synthesis of aryl β-D-glucoside, CAZy-annotated GT15, $Man_3GlcNAc_2$-*PP*-dolichol α–1,2-mannosyltransferase (EC 2.4.1.131) atalyzes the synthesis of oligosaccharide, CAZy-annotated GT22 $Man_6GlcNAc_2$-*PP*-dolichol α–1,2-mannosyltransferase (EC 2.4.1.259) atalyzes the synthesis of dolichyltetradecasaccharide, CAZy-annotated GT35 glycogen phosphorylase (EC 2.4.1.1) can directly degrade starch, CAZy-annotated GT31 β–1,3-galactosyltransferase (EC 2.4.1.122) catalyzes the synthesis of oligosaccharides, CAZy-annotated GT48 1,3-β-glucan synthase (EC 2.4.1.34) catalyzes the synthesis of glucan, CAZy-annotated GT20 α,α-trehalose-phosphate synthase (EC 2.4.1.15) catalyzes the synthesis of α,α-trehalose 6-phosphate, CAZy-annotated AA2 lignin peroxidase (EC 1.11.1.14) can directly degrade lignin (http://www.cazy.org/). This may be due to the fact that the stack fermentation stage is a glycosylation stage, so the microbial community has strong hydrolysis and synthesis capabilities. These results revealed that during the stack fermentation stage, a significant number of polysaccharide substrates undergo hydrolysis, leading to metabolite production that plays a pivotal role in the formation of Baijiu flavor. However, the relative abundance of GH170, GH73, GH2, GH31, GH25, GH1, GH13, GT51, GT2, and CBM50 were higher in fermented grain, compared to the stack fermentation material (Fig. 4B). CAZy-annotated GH73 mannosyl-glycoprotein endo-β-*N*-acetylglucosaminidase (EC 3.2.1.96) can directly degrade high-mannose glycopeptides and glycoproteins, CAZy-annotated GH2 exo-1,4-β-D-glucosaminidase (EC 3.2.1.165) can directly degrade chitosan, CAZy-annotated GH31 α-glucosidase (EC 3.2.1.20) can directly degrade starch, CAZy-annotated GH25 lysozyme (EC 3.2.1.17) can directly degrade peptidoglycan, CAZy-annotated GH1 β-glucosidase (EC 3.2.1.21) can directly degrade β-D-glucosides, CAZy-annotated GH13 glucan 1,4-α-maltohydrolase (EC 3.2.1.133) can directly degrade polysaccharides, CAZy-annotated GT51 peptidoglycan glycosyltransferase (EC 2.4.99.28) catalyzes the synthesis of peptidoglycan, CAZy-annotated GT2 cellulose synthase (EC 2.4.1.12) catalyzes the synthesis of cellulose (http://www.cazy.org/). These results indicated that CAZy families change during Baijiu fermentation, and they play an important role in the final production of flavor substances.

Functional gene category by blasting to eggnog database

Principal Coordinates Analysis (PCoA) of eggNOG functional levels revealed distinct category profiles across all

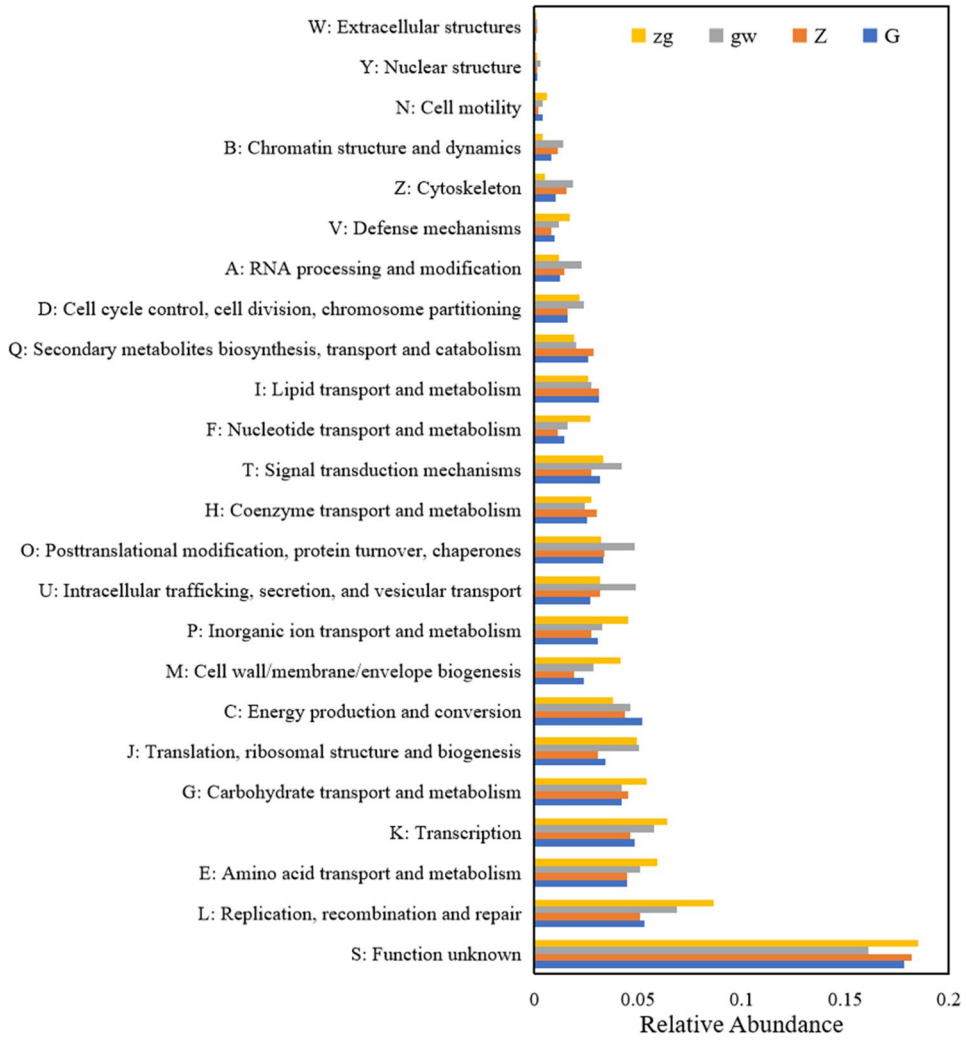

Fig. 5 Functional classification of annotated genes based on eggNOG database. Sample types: **G** (high-temperature Daqu), **Z** (medium-temperature Daqu), **gw** (stack fermentation material), and **zg** (fermented grain)

four sample types (Fig. S4). Genes were clustered into 24 eggNOG categories (Fig. 5), with seven dominant functions: S (Unknown), L (Replication/recombination/repair), E (Amino acid metabolism/transport), K (Transcription), G (Carbohydrate metabolism/transport), J (Translation/ribosomal biogenesis), and C (Energy conversion). Category S genes represent potentially novel functions from uncultured microorganisms, warranting further investigation. Categories L, K, J, and C support microbial proliferation and survival, while G and E drive core metabolic processes, confirming the critical roles of carbohydrate and amino acid metabolism in Daqu fermentation.

Notably, stack fermentation material exhibited elevated abundances of O (Posttranslational modification) and U (Intracellular trafficking) compared to those in traditional Daqu (Fig. 5), potentially reflecting enhanced microbial activation during two-day stacking. Conversely, fermented grain showed increased P (Inorganic ion transport) and M (Cell envelope biogenesis) abundances compared to stack fermentation material (Fig. 5), suggesting that these functions facilitate aromatic compound synthesis during fermentation.

Correlation between the microbiota, metagenomic function, and physicochemical properties

To better explore the changes of microflora function during Baijiu fermentation, water content, dry matter content, esterification power, saccharifying power, protease activity, and liquefaction power of samples were detected (Fig. 6). Physicochemical parameters serve as indicators of the fermentation status and the quality of the fermented grain. The esterification power, inherent in Daqu, is the capacity to produce esters post-fermentation, which is intricately linked to the production of flavor substances in Baijiu. The saccharifying and liquefaction

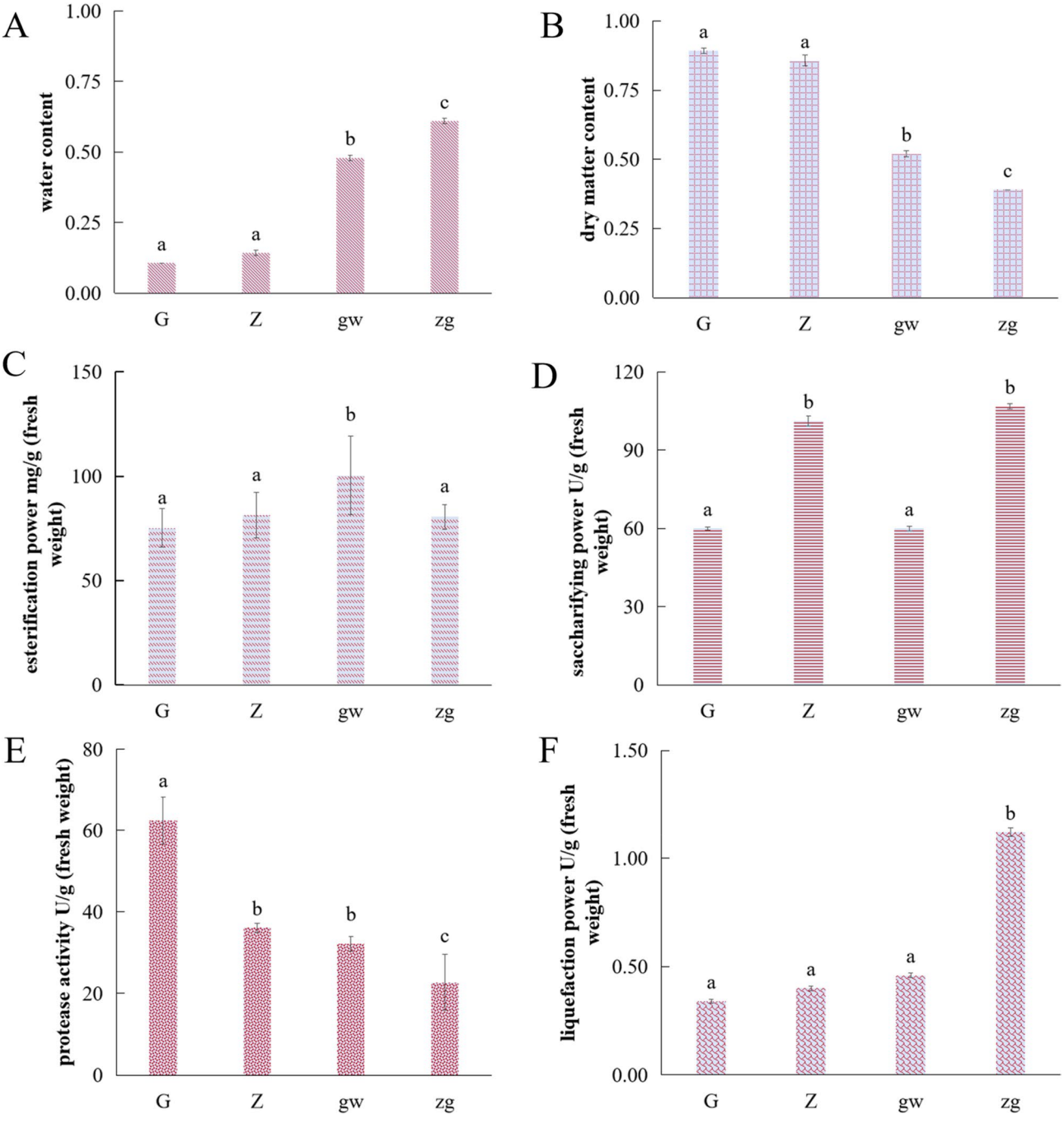

Fig. 6 Physicochemical properties of fermentation samples: **A** Water content, **B** Dry matter, **C** Esterification activity, **D** Saccharification activity, **E** Protease activity, **F** Liquefaction activity. Samples: **G** (high-temperature Daqu), **Z** (medium-temperature Daqu), **gw** (stack fermentation material), and **zg** (fermented grain). Distinct superscript letters (**a**, **b**, **c**) indicate significant differences ($p < 0.05$)

powers are associated with the hydrolysis of starch and cellulose in grains. Furthermore, protease activity plays a significant role in the breakdown of proteins and the release of amino acids during the brewing process, significantly impacting the quality and flavoring taste of Baijiu products.

There was no significant difference between high-temperature Daqu and medium-temperature Daqu in terms of water content, dry matter, esterification activity, and liquefaction activity. The saccharifying power of medium-temperature Daqu (101.20 ± 1.85 U/g) was significantly higher than that of high-temperature Daqu (60.00 ± 0.58 U/g) (Fig. 6D). Bacteria were more abundant in high-temperature Daqu, while eukaryotes were more abundant in medium-temperature Daqu (Fig. S1). The proportion of eukaryotes in medium-temperature

Daqu was higher than high-temperature Daqu (Fig. S1). Eukaryotes tend to have higher hydrolysis properties than bacteria for substances such as cellulose and starch [47]. Furthermore, *P. variotii* and *A. chevalieri* were more abundant in medium-temperature Daqu (Fig. 2C), and they can produce xylanolytic and cellulolytic enzymes [48, 49]. Therefore, we speculate that this might be the reason for the high saccharifying power of medium-temperature Daqu. These results were also shown by the fact that the GH relative abundance in medium-temperature Daqu was higher than in high-temperature Daqu (Fig. 4A). Meanwhile, CAZy-annotated GH31 α-glucosidase (EC 3.2.1.20) can directly degrade starch, and their expression correlates with saccharifying power ($r = 0.91$, $P = 0.044$). The protease activity of high-temperature Daqu (62.47 ± 5.84 U/mg) was significantly higher than that of medium-temperature Daqu (36.10 ± 1.13 U/mg) (Fig. 6E). CAZy-annotated GT35 glycogen phosphorylase (EC 2.4.1.1) can directly degrade starch, and their expression correlates with protease activity ($r = 0.99$, $P = 0.001$). These results indicated that the high content of GT 35 may be one of the reasons for the high protease activity of high-temperature Daqu (Fig. 4B).

The water content of fermented grain was significantly higher than that of stack fermentation material, while the dry matter content of fermented grain was significantly lower (Fig. 6A and B). This was attributed to microbial fermentation that produces water during the process of fermentation. The esterification power and protease activity of fermented grain (80.50 ± 6.02 mg/g and 22.71 ± 6.87 U/mg) were significantly lower than those of stack fermentation material (100.37 ± 9.20 mg/g and 32.23 ± 1.80 U/mg). CAZy-annotated GH35 1,3-β-galactosidase (EC 3.2.1.23) can directly degrade galactan ($r = 0.91$, $P = 0.043$), CAZy-annotated GH16 1,3-β-D-glucosidase (EC 3.2.1.39) can directly degrade glucan ($r = 0.95$, $P = 0.024$), CAZy-annotated GH76 1,6-α-mannosidase (EC 3.2.1.101) can directly degrade mannan ($r = 0.94$, $P = 0.031$), CAZy-annotated GT48 1,3-β-glucan synthase (EC 2.4.1.34) catalyzes the synthesis of glucan ($r = 0.96$, $P = 0.018$), CAZy-annotated GT15 $Man_3GlcNAc_2$-*PP*-dolichol α−1,2-mannosyltransferase (EC 2.4.1.131) atalyzes the synthesis of oligosaccharide ($r = 0.97$, $P = 0.014$), CAZy-annotated GT22 $Man_6GlcNAc_2$-*PP*-dolichol α−1,2-mannosyltransferase (EC 2.4.1.259) atalyzes the synthesis of dolichyltetradecasaccharide ($r = 0.94$, $P = 0.031$), and CAZy-annotated AA2 lignin peroxidase (EC 1.11.1.14) can directly degrade lignin ($r = 0.94$, $P = 0.029$), and their expression correlate with esterification power. These results indicated that the high content of GH35, GH16, GH76, GT48, GT15, GT22, and AA2 may be one of the reasons for the high esterification power of stack fermentation material (Fig. 4B). CAZy-annotated GT35 glycogen phosphorylase (EC 2.4.1.1) can directly degrade starch, and their expression correlates with protease activity ($r = 0.99$, $P = 0.001$). These results indicated that the high content of GT35 may be one of the reasons for high protease activity of stack fermentation material (Fig. 4B). However, the saccharifying power and liquefaction power of fermented grain (106.8 ± 1.07 mg/g and 1.12 ± 0.02 U/g) were significantly higher than those of stack fermentation material (60 ± 0.95 U/g and 0.46 ± 0.01 U/g). CAZy-annotated GH73 mannosyl-glycoprotein endo-β-*N*-acetylglucosaminidase (EC 3.2.1.96) can directly degrade high-mannose glycopeptides and glycoproteins ($r = 0.96$, $P = 0.022$), CAZy-annotated GH25 lysozyme (EC 3.2.1.17) can directly degrade peptidoglycan ($r = 0.94$, $P = 0.030$), CAZy-annotated GH1 β-glucosidase (EC 3.2.1.21) can directly degrade β-D-glucosides ($r = 0.96$, $P = 0.019$), and CAZy-annotated GH13 glucan 1,4-α-maltohydrolase (EC 3.2.1.133) can directly degrade polysaccharides ($r = 0.98$, $P = 0.010$), and their expression correlate with liquefaction power. These results indicated that the high content of GH73, GH25, GH1, and GH13 may be one of the reasons for the high liquefaction power of fermented grain (Fig. 4B). These results also showed that with the consumption of carbon sources in fermented grain, microorganisms tended to increase the saccharification and liquefaction power to obtain more carbon sources, while decreasing the esterification power and protease activity to reduce the secretion of products.

Conclusion

Fueled by consumer demand for novel flavors and health-conscious beverages, Baijiu research continues to evolve. We investigated Jiang-Nong Jianxiang Baijiu, which employs high-temperature Daqu and medium-temperature Daqu as its fermentation starter. This study provides a comprehensive metagenomic characterization of microbial communities and function of high-temperature Daqu and medium-temperature Daqu. We used metagenomic sequencing to reveal the microbial community dynamics and functional shifts during the Jiang-Nong Jianxiang Baijiu fermentation stage with high-temperature Daqu and medium-temperature Daqu. Our results will provide a theoretical reference for developing rationally designed standardized starter cultures, applying core synergistic microbial consortia and their functional traits, and optimizing precise fermentation management strategies at key brewing stages to enhance Baijiu quality. Future research should functionally validate the roles of key microbes and genes through their isolation and targeted experimentation, and construct synthetic microbial communities for precise fermentation control and fundamental study.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-025-12045-3.

Supplementary Material 1.

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Authors' contributions
LC, HS, YW, ZW, and EL wrote the main manuscript text. JZ, YW, JY, YZ, NC, SH, XL，LT, and WM processed some data of the manuscript. All authors reviewed the manuscript.

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Data availability
Metagenomic data were submitted to NCBI, SRA ID of the high-temperature Daqu (G) is SRR30592996, SRA ID of the medium-temperature Daqu (Z) is SRR30593003, SRA ID of the stack fermentation material (gw) is SRR30593127, and SRA ID of the fermented grain (zg) is SRR30593128. And all the data used to support the findings of this study are available from the corresponding author upon request.

Declarations

Ethics approval and consent to participate
There are no ethical issues involved in this study.

Consent for publication
Not Applicable.

Competing interests
The authors declare no competing interests.

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